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IMMUNOCHEMICAL STUDIES ON RABBIT ANTI-ANTIBODY[1]

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In 1956, Milgrom *et al.* described a thermostable agglutinating factor, anti-antibody (A-A), in human serum capable of agglutinating Rh+ red cells sensitized by incomplete anti-Rh antibodies (1, 2). In contrast to rheumatoid factors, A-A could not be neutralized by normal or immune human serum or by the γ-globulin fractions thereof. Subsequently rabbit sera were found with similar agglutinating properties for rabbit G+ red blood cells sensitized by incomplete anti-G of rabbit origin (3). Although the agglutinating capacity of rabbit A-A for G+ sensitized cells was not inhibited by prior exposure to rabbit γ-globulin, the agglutinating activity was completely removed by immune complexes composed of rabbit antibody and homologous antigen, e.g., bovine serum albumin (BSA) and rabbit anti-BSA. Accordingly, it was suggested that A-A was a specific reagent capable of distinguishing immune globulin bound to homologous antigen from "free" (unbound) γ-globulin, either with or without known antibody specificity; it was further suggested that the serologic reactions obtained with A-A provided evidence for structural modification of antibodies during their reaction with the corresponding antigens (3).

The present investigations were designed to delineate further the nature and specificity of rabbit A-A. Reactivity of A-A with nonspecifically aggregated γ-globulin, with proteolytic fragments of γ-globulin and with soluble antibody-hapten complexes were studied.

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MATERIALS

Two rabbit sera (10 and 1201) with high A-A titer were used in these investigations. The properties of serum 1201 (titer 1:128) have been described elsewhere (3); serum 10 displayed similar serologic behavior and agglutination titer. The incomplete rabbit anti-G serum used in these studies (titer 1:1000 by indirect antiglobulin test) was obtained from a G (-) rabbit immunized with G+ red cells.

Human, bovine and rabbit 7 S γ-globulins (HGG, BGG and RGG) were purchased from Pentex, Inc. (Cohn fraction II). Bovine serum albumin (BSA) and twice recrystallized preparations of mercuripapain and pepsin were obtained commercially. Rabbit anti-BSA was prepared by immunization of rabbits with the antigen incorporated in Freund's adjuvant. The γ-globulin was isolated from the serum by the sodium sulfate method of Kekwick (4).

Rabbit antibody to dinitrophenyl-BGG ("anti-DNP") was produced by immunization with DNP-BGG. This antiserum, the "homologous antigen" (DNP-BSA with 15 DNP groups/mole), univalent hapten (ε-DNP-lysine), and bivalent hapten (α,ε-bis DNP-lysine), were a gift of Dr. Z. Ovary, New York University.

METHODS

1. Agglutination. Thrice-washed red blood cells of G+ rabbits were sensitized by incubation of 2% cell suspensions in saline with an equal volume of a 1:30 dilution of the rabbit anti-G serum at 37°C for ½ hr. After three washings with 20- to 40-fold volumes of isotonic saline, the sensitized cells were resuspended in saline to provide a 1% suspension for use in subsequent agglutination experiments (5). Serial 2-fold dilutions of A-A sera and of control rabbit sera, either absorbed with immune complexes or unabsorbed, were titrated against sensitized and control (non-sensitized) red cells by the serial two-step dilution

method. Pipets were changed at each dilution step to prevent carry over. After incubation for 1 hr at room temperature, the mixtures were centrifuged and read for agglutination.

2. Inhibition of agglutination. To 0.1 ml of a solution containing 10 agglutinating units of A-A, equal volumes of various dilutions of inhibiting substances were added. The tubes containing the mixtures were left for 30 min at room temperature; then 0.1 ml of the suspension of sensitized rabbit erythrocytes was added to each tube. After 1 hr of further incubation at room temperature the tubes were centrifuged and inspected for agglutination. In most instances duplicate "blindfold" experiments were performed—(one set in Buffalo and one in San Francisco) with very similar results. (In most instances no more than one tube difference in agglutination or inhibition end points was found.)

3. Chromatographic separation of sera. The 7 S and 19 S containing fractions of A-A sera were separated by chromatography on diethylaminoethyl (DEAE)-cellulose, according to the methods of Pike and Schulze (6). Four major peaks were obtained, and analyzed by microimmunoelectrophoresis (7) with goat and sheep antisera to rabbit serum proteins. Peak I, the fall-through peak, (0.01 M phosphate, pH 8.0) contained only 7 S γ-globulins, whereas peak IV contained the bulk of the 19 S γ-globulins along with other serum proteins but was devoid of 7 S γ-globulin. Peaks II and III contained 7 S γ-globulins and other serum proteins. Before tests for activity, each chromatographic fraction was concentrated to a volume approximately twice that of the original serum sample and then dialyzed against normal saline.

4. Proteolytic fragments of γ-globulin. Papain fragments I, II, and III of rabbit 7 S γ-globulin were obtained by the method of Porter (8). The 5 S derivative was prepared and isolated by pepsin digestion followed by sodium sulfate precipitation (9). The presence of significant contamination by undegraded materials was excluded by agar diffusion methods (7, 10), using goat and sheep antisera to rabbit 7 S γ-globulin, and in some instances by analytic ultracentrifugation.

5. Aggregation of γ-globulin. Rabbit 7 S γ-globulin and its various proteolytic fragments were aggregated by the methods of Talmadge (ethanol)

TABLE I

Reaction of diethylaminoethyl (DEAE)-cellulose column fractions[a] of anti-antibody with rabbit G+ red cells sensitized by incomplete anti-G

	Serum Proteins in Fractions[b]	Dilution of Fractions or Sera								
		Sensitized cells							Nonsensitized cells	
		3	9	27	81	243	729	Saline	3	9
A-A serum 1201										
Fraction I.....	7 S γ	0	0	0	0	0	0	0	0	0
Fraction II.....	7 S γ	0	0	0	0	0	0	0	0	0
Fraction III....	7 S γ, γ_{1A} and β-globulins	0	0	0	0	0	0	0	0	0
Fraction IV....	19 Sγ, albumin, α- and β-globulins	+++	++	+	0	0	0	0	±	0
Whole serum...	All	+++	+++	++	++	++	±	0	0	0
Normal rabbit sera										
Fraction I.....	7 S γ	0	0	0	0	0	0	0	0	0
Fraction II.....	7 S γ	0	0	0	0	0	0	0	0	0
Fraction III....	7 S γ-, γ_{1A}- and β-globulins	0	0	0	0	0	0	0	0	0
Fraction IV....	19 Sγ, albumin, α- and β-globulins	0	0	0	0	0	0	0	0	0
Whole serum...	All	0	0	0	0	0	0	0	0	0

[a] Each original fraction represents a 2-fold dilution of original sera.

[b] As determined by immunoelectrophoresis.

(11) and of Ishizaka (bis-diazotized benzidine (BDB)) (12), as modified by Taranta (13).

6. Absorption experiments. Absorption experiments were performed with precipitates composed of BSA and either 7 S or 5 S rabbit anti-BSA. Precipitates were made in slight antibody excess by mixing appropriate amounts of 5 S or 7 S antibody γ-globulin (approximately 0.5 mg/ml of antibody protein) with 20 or 40 μg of BSA and incubating the mixtures at 37°C for 1 hr followed by 4°C for 7 days. The precipitates were twice washed, then suspended in sufficient volumes of saline to provide an antibody nitrogen concentration of 240 and 160 μg/ml for the 7 S and 5 S precipitates, respectively; 4-fold saline dilutions of the two precipitate suspensions were then prepared and 0.25 ml of each solution added to an equal volume of a 1:10 dilution of A-A serum. After thorough mixing, the solutions were incubated at 37°C for 1 hr, then at 4°C for 16 hr. As controls, mixtures of A-A and pooled normal rabbit Cohn Fraction II (0.1–1.0 mg), with or without BSA (20 μg–1.0 mg), were similarly treated. After incubation, the mixtures were centrifuged at 4°C and the supernatants titrated in the usual way against sensitized G+ red cells.

RESULTS

Table I indicates that the A-A activity of serum 1201 was confined to the 19 S-containing fractions obtained by DEAE column chromatography. The 7 S- and 19 S-containing fractions similarly obtained from normal rabbit sera were devoid of such activity, indicating that the separation process *per se* was not responsible for the activity in the 19 S-containing peak of the A-A serum. The 19 S nature of the A-A was also demonstrated by the abolition of agglutinating activity upon treatment of whole serum (or of the "19 S-containing fraction") with 0.1 M mercaptoethanol, which destroys the activity of 19 S, but not of 7 S, antibodies (14, 15).

Treatment of pooled rabbit γ-globulin by methods previously shown to produce aggregation (11, 13) produced sufficient structural alteration to result in development of reactivity with A-A, as evidenced by inhibition of the standard A-A test system (Table II).

Table II also demonstrates the effect of the Porter papain fragments of rabbit γ-globulin on the agglutinating system composed of rabbit A-A 1201 and the sensitized rabbit red blood cells. As shown, Fragments I and II possessed significant inhibitory activity whereas Fragment III failed to exhibit such activity. Similar results were obtained with A-A serum 10 and with the 19 S fraction of A-A serum 1201 in that only Fragments I and II inhibited the agglutination. The effect obtained with a precipitate of human serum and a rabbit antiserum thereto are also shown for comparative purposes. BDB aggregation of Fragments I and II increased their inhibitory capacity

TABLE II

Inhibition of the agglutination of sensitized G+ red blood cells by anti-antibody

Inhibitor	Dilution of Inhibitors[a]						
	1:1	1:2	1:4	1:8	1:16	1:32	
Rabbit γ-globulin							
Untreated	+++	+++	+++	++	+++	+++	
Aggregated							
BDB[b]	−	−	±	±	+++	+++	
Ethanol	−	−	±	±	+++	+++	
Immune precipitate[c]	−	−	−	−	−	−	
Papain fragments							
Fragment I	−	−	±	±	++	+++	
Fragment II	−	−	±	±	+++	+++	
Fragment III	+++	+++	+++	+++	+++	+++	
Saline buffer							+++

[a] Concentration of initial dilution of each inhibitor: 5 mg/ml.

[b] Bis-diazotized-benzidine.

[c] Obtained by mixing human serum (1:100 dilution) with undiluted rabbit antiserum against human serum.

TABLE III

Anti-antibody titers[a] after absorption[b] with immune precipitates

Absorbing Agent	Dilutions of Anti-Antibody Serum 1201					
	20	40	80	160	320	Saline
Saline control	+++	+++	+++	++	tc[c]	0
7 S anti-BSA + BSA						
a) (60[d] γ)	±	0	0	0	0	0
b) (15 γ)	++	+	+	0	0	0
c) (0.05 γ)	+++	++	++	±	0	0
5 S anti-BSA + BSA						
a) (40[d] γ Ab N)	±	0	0	0	0	0
b) (10 γ Ab N)	+	+	+	tc	0	0
c) (0.167 γ)	++	++	++	±	tc	0
7 S anti-BSA (60[d] γ)	+++	+++	++	±	tc	0
Rabbit Cohn Fraction II + BSA						
a) (160[e] γ)	+++	+++	++	±	tc	0
b) (16 γ)	+++	+++	+++	+	0	0
Porter Fragment III						
(60[e] γ + BSA (20 γ))	+++	+++	+++	±	tc	0

[a] Agglutination reactions with G+ cells sensitized by incomplete anti-G.
[b] A-A absorbed with equal volume of solution containing serum #1201.
[c] Trace.
[d] γ antibody nitrogen.
[e] γ of protein N.

slightly (1 to 2 tubes), but similar treatment of Fragment III did not result in appearance of inhibitory activity for A-A. Heating the fragments at 63°C for 15 min did not change the results significantly.

The activity of Fragments I and II with A-A was also demonstrated by absorption experiments (Table III). Precipitates formed by 5 S rabbit antibody (lacking Fragment III) proved as effective as those formed by 7 S antibody in absorbing A-A activity from serum 1201. Absorption with equivalent amounts of antigen, or with antiserum in the absence of antigen, failed to diminish A-A activity. In simultaneous experiments the precipitates formed with 5 S anti-BSA produced no detectable change in titer of two rheumatoid sera in a serologic test system utilizing human Rh_o red cells coated with anti-Rh_o antibody (16).

Effect of combination of antibody γ-globulin with antigen in nonprecipitating systems. To study the effect of soluble immune complexes on A-A activity, A-A sera were absorbed with complexes of hapten and corresponding antibody. In preliminary experiments, precipitation reactions were performed by adding undiluted anti-DNP serum to varying concentrations of DNP-BSA. Table IV indicates that mixtures of anti-DNP sera with quantities of DNP-BSA sufficient to cause visible precipitation produced marked inhibition of A-A agglutinating activity for G+ sensitized cells. From the results listed in Table IV, a concentration of DNP-BSA capable of producing maximal precipitation (0.25 mg/ml) was selected for further experiments. Attempts were made to inhibit the precipitation of DNP-BSA at this concentration by adding univalent (ϵ-DNP-lysine) or bivalent (α,ϵ-bis DNP-lysine) hapten to the anti-DNP serum; both of these are incapable of precipitating with anti-DNP-BSA as shown previously by Ovary (17). The results are illustrated in Table V. It is evident that concentrations as low as 0.1 mg/ml of univalent hapten or 0.03 mg/ml of bivalent hapten were capable of inhibiting the anti-DNP-DNP reaction, thus indicating combination of anti-DNP with haptens.

Mixtures of anti-DNP with optimal amounts of DNP-BSA, univalent hapten, or bivalent hapten were then added to tubes containing 1:10 dilutions of A-A sera. In contrast to the results obtained with anti-DNP and DNP-BSA, the antibody-hapten mixtures were incapable of in-

TABLE IV

Reaction of anti-DNP with DNP-BSA

	Concentration of DNP-BSA (mg/ml)									
	13	1.3	0.25	0.13	0.065	0.032	0.013	0.0013	0.00013	Saline
Precipitation[a]	0	+	+++	++	+	+	0	0	0	0
Inhibition of A-A 10[b]	±	±		0		0	0	+	++	++

[a] 0.1 ml undiluted anti-DNP, 0.1 ml solution of DNP-BSA, 0.1 ml saline.

[b] 0.1 ml undiluted anti-DNP, 0.1 ml solution of DNP-BSA added to agglutinating system (0.1 ml of 1:20 dilution of A-A 10 plus 0.1 ml of sensitized G cells).

TABLE V

Effect of hapten-anti-hapten complexes on anti-antibody

	Concentration of Hapten (mg/ml)										
	1.0	0.25	0.1	0.06	0.03	0.015	0.010	0.004	0.0013	0.00013	Saline
Inhibition of precipitation of anti-DNP[a] with DNP-BSA											
ϵ-DNP-lysine	0	0	0		++		++	++	++	++	++
α,ϵ-bis DNP-lysine	0	0	0		0		+	++	++	++	
Modification of anti-DNP (inhibition of A-A 10)[b]											
ϵ-DNP-lysine	++	++		++		++		++	++	++	++
α,ϵ-bis DNP-lysine	++	++		++		++		++	++	++	
Positive control (DNP-BSA)			0		0	+			++	++	

[a] 0.1 ml undiluted anti-DNP-BGG, 0.1 ml of hapten solution and 0.1 ml of DNP-BSA (0.25 mg/ml).

[b] Inhibition of agglutination of G+ sensitized cells by anti-DNP-hapten complex (0.1 ml anti-DNP-BGG, 0.1 ml hapten solution; 0.1 ml of A-A 10 (1:20 dilution) and 0.1 ml of sensitized cells).

hibiting A-A activity, although the haptens were employed at concentrations previously shown to cause inhibition of the precipitating system.

DISCUSSION

On the basis of their serologic properties, it was suggested that anti-antibodies (A-A) are specific reagents which detect structural modifications in antibody resulting from reaction with the corresponding antigen (1–3). Subsequently, immunochemical data provided by Robert and Grabar (18), and Ishizaka and Campbell (19) furnished additional evidence that structural modifications of antibody are produced during immunologic reactions. The former authors demonstrated liberation of SH groups as a result of antigen-antibody interaction; the latter authors found increased levorotation as a result of antibody combination with antigen.

The data provided in the above experiments with anti-DNP antibody suggest that molecular alteration of the antibody portion of the immune complex to a degree sufficient to be detectable by reaction with anti-antibody occurs only when lattice formation takes place: Apparently, alterations, if any, resulting from formation of short or linear complexes are insufficient to produce reactivity with A-A. Thus, the precipitating system of anti-DNP-DNP-BSA was capable of inhibiting A-A. In contrast, the nonprecipitating complexes of univalent or bivalent hapten with anti-hapten antibody (anti-DNP) failed to inhibit A-A activity. Similarly, the addition of sufficient BSA to anti-BSA to form soluble complexes in antigen excess results in increased levorotation, whereas formation of a hapten-anti-hapten complex does not change the optical rotation of antibody (20).

Structural modifications of rabbit γ-globulin produced by nonimmunologic methods, e.g., treatment by BDB (13) or by cold ethanol (11), also resulted in reactivity with A-A. It is of interest that γ-globulin so treated acquires biologic properties manifested by antibodies as a consequence of reaction with antigen, e.g., ability to fix complement, to produce wheal and erythema reactions, and to elicit Arthus phenomenon (20). Hence, structural alteration of γ-globulin either by immunologic reaction or by certain nonspecific methods leads to the acquisition of various biologic properties as well as the ability to react with A-A. However, the appearance of these properties is not due to modification at the same structural site. The ability to fix complement and to produce skin reactions is a result of alteration of Fragment III (13, 20). In contrast, the data shown here indicate that the specific sites reactive with A-A are absent from Fragment III, as demonstrated by absorption experiments with precipitates formed with 5 S and 7 S antibodies (Table III) and also by inhibition experiments with the 3.5 S fragments (Table II). The latter experiments indicate that the specific antigenic sites reactive with A-A are present in Fragments I and II, but not in III.

The limitation of these "antigenic sites" to Fragments I and II carries certain implications. First, since reactivity is directed against the fragments of the parent molecule containing the antibody combining sites, the relation of the combining site to the "antigenic site" for anti-antibody merits further investigation. Secondly, the inhibiting activity of Fragments I and II for A-A in the absence of combination with antigen suggests either that a) the proteolytic process *per se* results in structural modifications, or b) that "buried" antigenic determinants in I and II are exposed by papain digestion. In either case, these structural alterations appear similar to those induced by immunologic reactions or nonspecific aggregation. Third, reactivity of Fragments I and II suggests the possibility that similar antigenic sites for A-A may be present in γ_{1A} and 19 S γ-antibodies as well, since the antigenic determinants (antigenic groups I and II) shared in common by the three immune globulins are restricted to the univalent fragments of 7 S γ-globulin (21). Further, the shared genetic factors (Inv factors) are also restricted to the univalent fragments (22, 23).

The delineation of rabbit "A-A" as 19 S γ-globulin reactive with red cells sensitized by homologous 7 S "incomplete" antibody of the same species superficially suggests that A-A might be the rabbit analogue of human rheumatoid factors. However, the rheumatoid factor agglutinating system displays serologic specificity for genetically determined iso-antigens in human 7 S γ-globulin (16, 24); no such specificity of "A-A" for genetic factors in rabbit γ-globulin has thus far been detected (3, 25). Further, the agglutinating system established by rheumatoid factor and human red cells sensitized by anti-Rh antibody can be inhibited by pooled human γ-globulin, whereas the reactions of rabbit "A-A" with sensitized G+ rabbit red cells cannot be inhibited by pooled rabbit γ-globulin. Also rheumatoid factor reacts only with the nonantibody papain fragment of rabbit or human γ-globulin (26–28); in contrast, A-A, as shown here, reacts only with the univalent fragments (I and II) of rabbit γ-globulin; in addition, absorption with precipitates produced by 5 S antibodies (lacking Fragment III) markedly diminishes the agglutinating activity of A-A sera but fails to decrease the titer of rheumatoid sera in comparable serologic test systems utilizing human reagents. Hence, it would appear that A-A and rheumatoid factor are two distinct entities. It seems not unlikely, however, that these two serum factors represent manifestations of rather closely related biologic phenomena, and it is probable that anti-γ-globulin factors with intermediate properties (e.g., specificity for genetic determinants in aggregated γ-globulin), difficult to label by other names, will be delineated (29). Before final conclusions as to the significance of the various anti-γ-globulin factors are drawn, further studies on their origin are clearly warranted.

SUMMARY

Anti-antibody in rabbits has been found to be a 19 S globulin with specificity for rabbit 7 S γ-globulin altered either by combination with antigen or by certain nonspecific methods. The anti-antibody combines with antigenic determinants on Fragments I and II, but not III, of the papain-digested 7 S γ-globulin molecule. The implications of these findings are discussed.

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